METHODS

Animals

Male and female mice with null mutation of the α5 nAChR subunit gene *Chrna5* (α5 knockout) and their wildtype littermates were bred in our animal facilities. Brain structure and baseline behavioral measures between the knockout mice and wildtype littermates have been previously characterized [1]. The mutant mice have been bred for more than 10 generations onto a C57BL6 background. Breeding was conducted by mating heterozygous pairs. All mice were housed in cages of 1-3 and were at least 6 weeks of age at the beginning of each experiment. Male Wistar rats weighing 275-300g were purchased from Charles River Laboratories and housed 1-2 per cage. Mice and rats were maintained in an environmentally controlled vivarium on a 12h:12h reversed light:dark cycle, and food and water were provided *ad libitum* until behavioral training commenced. During self-administration procedures, mice and rats were food restricted to 85-90% of their free-feeding body weight, but water was maintained without restriction. All procedures were conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute - Florida.

Genotyping

Around 21 days of age, mouse pups were weaned and their tails were clipped for genetic analysis. DNA was extracted with a tissue DNA extraction kit purchased from Biomiga, Inc. (San Diego, CA). Primers for the α5 wildtype and mutant genes

were: α5 wildtype forward (5'-CACTGTCACTTGGACGCAGCC-3'); α5 wildtype reverse (5'-GTTCCCCTTGCTCCCCATTGC-3'), Neo-1 (5'-CTTTTTGTCAAGACCGACCTGTCCG-3'); and Neo-2 (5'-CTCGATGCGATGTTTCGCTTGGTG-3'). Samples were processed for genetic amplification with PCR and subsequently run on a 1% agarose gel with ethidium bromide. The band for the α5 wildtype gene was at 190bp, and the α5 mutant gene was at 290bp.

Drugs

For self-administration experiments in mice and rats, (-)-nicotine hydrogen tartrate salt (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.9% sterile saline. All doses of nicotine refer to the free-base form. The NMDA antagonist LY235959 (Tocris, Ellisville, MO) or lidocaine (2%, Sigma Chemical Co.) were microinjected at a volume of 0.5 μl for over 1 min, and the injector remained in place for an additional 2 min to allow for diffusion. The pH of solutions was adjusted to ~7.4.

Surgery

Mice and rats were anesthetized with an isoflurane (1-3%)/oxygen vapor mixture and prepared with intravenous catheters. Briefly, the catheters consisted of a 6 cm (mice) or 12 cm (rats) length of silastic tubing fitted to guide cannula (Plastics One, Wallingford, CT) bent at a curved right angle and encased in dental acrylic. The catheter tubing was passed subcutaneously from the animal's back to the right jugular vein, and a 1 cm (mice) or 2.5 cm (rats) length of the catheter tip was

inserted into the vein and tied with surgical silk suture. Catheters were flushed daily with physiological sterile saline solution (0.9% w/v) containing heparin (10-60 USP units/ml). Catheter integrity was tested with the ultra short-acting barbiturate anesthetic Brevital® (methohexital sodium, Eli Lilly, Indianapolis, IN).

Intravenous self-administration

Mice and rats were mildly food restricted to 85-90% of their free-feeding body weight and trained to press a lever in an operant chamber (Med Associates, St. Albans, VT) for food pellets (20 mg pellets mice; 45 mg food pellets rats; TestDiet, Richmond, IN) under a fixed-ratio 5, time out 20 sec (FR5TO20 sec) schedule of reinforcement prior to catheter implantation. Once stable responding was achieved (>25 pellets per session in mice; >90 pellets per session in rats), subjects were catheterized as described above. The animals were allowed at least 48 h to recover from surgery, then permitted to respond for food reinforcement again under the FR5TO20 sec schedule. Once food responding criteria was reestablished, subjects were permitted to acquire intravenous nicotine self-administration by autoshaping during 1 h daily sessions, 7 days per week. Nicotine was delivered through the tubing into the intravenous catheter by a Razel syringe pump (Med Associates). Each nicotine self-administration session was performed using 2 retractable levers (1 active, 1 inactive) that extend 1 cm into the chamber. Completion of the response criteria on the active lever resulted in the delivery of an intravenous nicotine infusion (0.03 ml infusion volume for mice; 0.1 ml for rats). Responses on the inactive lever were recorded but had no scheduled consequences. For dose-

response studies (fixed and progressive ratio schedules), animals were presented with each dose of nicotine for at least 5 days (mice) or 3 days (rats); the mean intake over the last 3 (mice) or 2 (rats) sessions for each dose was calculated and used for statistical analysis. Nicotine doses were presented in ascending order with saline last (mice; see Ref.[2]) or according to a within-subjects Latin square design (rats). In between each dose, subjects were placed back on the training dose for at least 2 days or until their intake returned to baseline levels before being tested on the next dose.

Surgical procedures for microinjections and ICSS electrode placement

Animals were anesthetized as above and positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA). Unless otherwise noted, the incisor bar was set to the 'flat-skull' position. To test the efficacy of the re-expressing and knockdown viruses *in vivo*, bilateral injections were made into the hippocampus of mice or rats, respectively. This area was chosen based on the constitutive expression of α5 nAChR subunit mRNA in wildtype animals. In mice, six bilateral injections (1 μl each at a flow rate of 1 μl per min) were made at the following coordinates: anterior-posterior (AP): -1.7 mm from bregma; medial-lateral (ML): ±0.75 mm from midline; dorsal-ventral (DV): -2.05 mm, -1.80 mm and -1.3 5mm from brain surface [3]. In rats, the six hippocampal injections (three 2 μl injections per side at a flow rate of 1 μl per min) were made at the following coordinates: AP: -3.3 mm from bregma; ML: ±1.1 mm from midline; DV: -3.6 mm, -3.0 mm and -2.4 mm from brain surface [4]. For habenular injections in mice, the needle was angled 20° toward midline, and

bilateral injections (0.375 μl each) were administered at a rate of 0.375 μl per min. For habenular injections in rats, the lentivirus was injected bilaterally based on previously published coordinates [5]. The incisor bar was set to 5 mm above plane, and the injector needle was at a 10° angle toward midline (AP: -2.2 mm from bregma; ML: ±1.5 mm from midline; DV: -4.9 mm from brain surface). The bilateral injections (1 μl each) were administered at a rate of 1 μl per min. For all of the injections, the injector needle was remained in place for a minimum of 2 min post-injection. For IPN and VTA microinjections in rats, guide cannula (Plastics One) were implanted as follows: IPN (flat skull; 10° angle toward midline; AP: -6.72 mm from bregma; ML: ±1.6 mm from midline; DV: -6.5 mm from brain surface) or VTA (bilateral; flat skull; 6° angle toward midline; AP: -5.4 mm from bregma; ML: ±1.3 mm from midline; DV: -7.0 mm from skull) [4]. The MHb guide cannula coordinates were the same as for the lentiviral injections, except with DV at -2.9 mm from brain surface. For all of the cannula, injector needles extended 2 mm below the tip of the cannula for placement into the brain region. For the ICSS electrode, a stainless steel bipolar electrode (Plastics One) was implanted into the lateral hypothalamus (AP: -0.5 mm from bregma; ML: ±1.7 mm from midline; DV: -8.3 mm from brain surface) [4].

ICSS Behavioral Procedure

Rats were trained to respond according to a modification of the discrete-trial current-threshold procedure of Kornetsky and Esposito [6,7] in an operant box equipped with a wheel manipulandum and ICSS stimulator (Med Associates). Briefly, a trial was initiated by the delivery of a non-contingent electrical stimulus.

This electrical reinforcer had a duration of 500 ms and consist of 0.1 ms rectangular cathodal pulses that delivered at a frequency of 50-100 Hz. The frequency of the stimulation was selected for individual rats so that threshold elevation and lowering may be detected, and this frequency was held constant throughout the experiment. A one-quarter turn of the wheel manipulandum within 7.5 sec of the delivery of the non-contingent stimulation resulted in the delivery of an electrical stimulus identical in all parameters to the non-contingent stimulus that initiated the trial. After a variable inter-trial interval (7.5-12.5 sec, mean of 10 sec), another trial was initiated with the delivery of a non-contingent electrical stimulus. Failure to respond to the non-contingent stimulus within 7.5 sec resulted in the onset of the inter-trial interval. Responding during the inter-trial interval delayed the onset of the next trial by 12.5 sec. In each testing session, current levels were varied in alternating descending (x2) and ascending (x2) series in 5 μA steps. A set of five trials was presented for each current intensity. The threshold for each series is defined as the midpoint between two consecutive current intensities that yield "positive scores" (animals respond for at least three of the five trials) and two consecutive current intensities that yield "negative scores" (animals do not respond for three or more of the five trials). The overall threshold for the session is defined as the mean of the thresholds for the four individual series. Threshold data are presented as percent of baseline values due to inter-subject variability in baseline rates.

Generation of lentivirus

For α5 subunit re-expression studies, the mouse α5 nAChR subunit gene, *Chrna5*, was cloned into the pCDF1 lentivirus expression vector containing cop-GFP from Systems Biosciences, Inc. (Mountain View, CA). For α5 subunit knockdown studies, two different short hairpin interfering RNAs (shRNA) directed against the rat *Chrna5* gene were designed using the Genscript, Inc. online construct builder (see Supplementary Figs for shRNA sequence). The shRNAs were cloned into the pRNAT-U6.2/Lenti construct containing GFP (GenScript, Piscataway, NJ). Control vectors were identical to the expression constructs, but without the gene insert.

Generation of lentivirus

To generate lentivirus supernatant, HEK-293FT packaging cells (3.75×10^6 293TN cells per 10 cm plate) were transfected with the vectors, along with the pPACKF1TM Lentiviral Packaging Kit using lipofectamine reagent and plus reagent (Invitrogen) according to the manufacturer's instructions. Medium containing virus particles (~10 ml) was harvested 48–60 h post-transfection by centrifugation at 76,755g at room temperature for 5 min to pellet cell debris and filtered through 0.45 mm PVDF filters (Millex-HV). To concentrate the viral supernatant for administration, supernatants were centrifuged at 32,000g for 90 min at 4 °C, and the precipitate re-suspended in 100 μl cold PBS. Supernatants were aliquoted into 10 μl volumes and stored at −80 °C until use.

Estimation of lentivirus titer

Viral supernatant titers were determined using the Lentivector Rapid Titer Kit from System Biosciences, according to the manufacturer's instructions. The number of infectious units per ml of supernatant (IFU ml^{-1}) was calculated as follows: Multiplicity of infection (MOI) of the sample × the number of cells in the well upon infection × 1,000 / μl of viral supernatant used.

Tissue dissection

Mice and rats were euthanized by inhalation of CO_2, brains were rapidly removed, and frozen on dry ice. Tissues were stored at -80°C until dissection. Brains were sliced on a cryostat, and bilateral dissections were made for the hippocampus, habenula, IPN and/or VTA with a scalpel. Samples were pooled across multiple subjects due to the small size of selected brain areas and stored in at -80°C until processing for RNA isolation.

RNA Isolation and real-time RT-PCR

Cells grown in monolayer or dissected tissue was homogenized in RNA-STAT60 (Tel-Test Inc., Friendswood, TX), 250 μl of chloroform was added, and the samples were vortexed for 1 min. Samples were then centrifuged for 15 min at 12,000 x g at 4°C, and the upper aqueous RNA containing layer was removed for an additional RNASTAT60/chloroform extraction. The RNA was precipitated with 2 X volume of isopropanol overnight at -20°C and centrifuged for 30 min at 12000 X g. The RNA pellets were washed twice with 70% ethanol/RNAase-free water and subsequently resuspended in RNAsecure (Ambion/Applied Biosystems, Austin, TX), and ~10 μg of

RNA from each sample was treated with Turbo DNase (Ambion/Applied Biosystems) for 60 min at 37°C to degrade residual genomic DNA. To assess RNA levels, samples were reverse transcribed into cDNA with the TaqMan High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Thereafter, they were processed with the TaqMan Universal PCR kit with the mouse or rat *CHRNA5* gene expression assay (Applied Biosystems); controls consisted of either β-actin or 18S. Samples were quantified by real-time RT-PCR (7900 Real-Time PCR system; Applied Biosystems). All data were normalized in accordance with the mean housekeeping mRNA expressing levels as an internal control. Comparison between groups made using the method of $2^{-\Delta\Delta Ct}$.

Brain Perfusion and Fixation

Subjects were anesthetized with sodium pentobarbital (0.1 mg/10 g body weight) and perfused through the ascending aorta with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS; pH 7.4). Brains were harvested, postfixed for 2 hrs in 4% paraformaldehyde, and then stored in 30% sucrose in PBS. All brains were cut into 30-40 μm coronal sections on a microtome, and the floating sections were stored in 0.1 M PBS with 0.01% sodium azide at 4°C until processing for immunocytochemistry.

Fluorescence Immunolabeling

Floating sections were processed for GFP fluorescent immunostaining. To localize the GFP-tagged lentivirus-infected cells in mice, we utilized a rabbit polyclonal IgG

that recognizes GFP cloned from copepod *Pontellina plumata* (copGFP). To localize the lentivirus tagged with GFP in rats, we utilized a chicken polyclonal IgG that recognizes a 27kDa protein derived from the jellyfish *Aequorea Victoria*. Further, to identify IPN we utilized a guinea pig polyclonal IgG that recognizes VAChT. Sections were rinsed in 0.1M PBS, pH 7.4, with 0.3% Triton-X 100 (PBT) and then blocked in 10% normal donkey serum/PBT. Thereafter, sections were incubated in the primary antibody in PBT at 4°C overnight. The primary antibodies were diluted as follows: rabbit anti-copGFP (1:2,000; Evrogen, Moscow, Russia), chicken anti-GFP (1:2,000; Millipore, Billercia, MA) or guinea pig anti-VACHT (1:500; Millipore). On day 2, the sections were rinsed and incubated in Alexa 488 donkey anti-rabbit (1:400; Invitrogen), DyLight 488 donkey anti-chicken (1:400; Jackson ImmunoResearch, West Grove, PA) and/or DyLight 594 or 647 donkey anti-guinea pig (1:500; Jackson ImmunoResearch) secondary antibodies in 0.3% PBT for 2 hrs. Next, the sections were rinsed, mounted on slides with vectashield (with or without DAPI) (Vector Labs, Burlingame, CA), and coverslipped. Controls included processing the secondary antibodies alone to verify background staining, processing each primary with the secondary antibody to verify laser-specific excitation, examining for autofluorescence in an alternate laser channel with tissue lacking that laser-specific probe, and using sequential scanning. For subsequent fluorescent images, only the brightness and/or contrast levels were adjusted post-acquisition and were imposed across the entire image. To provide a complete view of multiple brain structures from the same section of lentiviral injected mice and rats, lower magnification images were merged.

$^{86}Rb^{+}$ Efflux

^{86}RbCl (average initial specific activity 15 Ci/mg) as well as Optiphase Supermix scintillation cocktail was purchased from Perkin-Elmer NEN (Boston, MA). The α5 knockout mice were injected with either the Lenti-CHRNA5 or Lenti-Control vector as previously described. Following an incubation period of at least 3 weeks, mice were killed synaptosomes generated from the IPN, habenula, hippocampus, striatum, thalamus and cortex as described previously [8]. Samples were loaded with $^{86}Rb^{+}$ and acetylcholine-stimulated $^{86}Rb^{+}$ efflux was measured as described previously [8], with each sample stimulated only once. $^{86}Rb^{+}$ efflux was expressed as the increase in signal above basal efflux. A non-linear least squares curve fit to a first order equation ($C_t = C_0 * e^{-kt}$), where C_t is the basal efflux counts at time, t, C_0 is the estimated efflux counts at t = 0 sec, and k is the first order decay constant) was used to estimate basal efflux for each sample. Counts in fractions preceding and following the peak were used for curve fitting. Acetylcholine-stimulated efflux was calculated by summing the counts in the fractions exceeding basal efflux during ACh exposure and dividing by the corresponding basal efflux counts. This value represents total peak relative to baseline.

Fos Procedure

Wildtype and α5 subunit knockout mice were injected subcutaneously with nicotine (0.5 or 1.5 mg/kg, free-base) or saline. The moderate dose of nicotine is known to be rewarding in these mice, reflected in the conditioning of a place preference [9]. The

higher dose of nicotine is aversive, reflected in the induction of a conditioned taste aversion in wildtype mice [10]. After 2 hr, each subject was perfused and brains were removed and stored as described above. Brain sections were cut at 30μm on a cryostat and stored in 0.1M PBS with 0.01% sodium azide until processing. For Fos immunolabeling, sections were rinsed in 0.1M PBS (ph 7.4), treated with 0.3% H_2O_2-PBS for 15 min, rinsed in PBS, and then blocked in 10% normal goat serum and 0.5% Triton X-100 in PBS for 1 hr. Thereafter, sections were incubated in rabbit anti-cfos IgG (1:500 dilution; Abcam, Cambridge, MA) in 0.5% Triton-PBS overnight at 4°C. The following day, sections were incubated at room temperature for 2 hrs, rinsed in PBS, and then incubated in 1:300 dilution of goat anti-rabbit secondary IgG (Vector Labs) in 0.5% Triton X-100 in PBS for 2 hrs. Following rinsing, sections were incubated in ABC Elite (Vector Labs) for 90 min, rinsed in PBS, and immunoreactivity was revealed by using 3-diaminobenzidine (DAB) with nickel (Vector Labs). To reduce variability in the background and to standardize the staining, sections from subjects across groups were processed concurrently. Sections were mounted and coverslipped with Permount (Fisher Scientific). Cell numbers and region volumes for the interpeduncular nucleus, ventral tegmental area and ventromedial hypothalamus were quantified under 40x magnification using unbiased stereological methods and the optical fractionator probe with Stereo Investigator software (MicroBrightField, Inc., Williston, VT). This method of assessing total volume and cell number has been validated and employed in many prior studies. Total cell counts and area measurements were determined for each

brain area, and cell density (number of cells per cubic millimeter) was calculated for each subject.

Statistical Analyses: All data were analyzed by one- or two-way analysis of variance (ANOVA) or t-test using Graphpad Prism software (La Jolla, CA). Significant main or interaction effects were followed by Bonferroni or Newman-Keuls post-hoc tests as appropriate. The criterion for significance was set at $p<0.05$.

Cited literature for methods

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